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(54) Title: LIGAND–PEG POST–COATING STABILIZED LIPOPLEX AND POLYPLEX FOR TARGETED GENE DELIVERY

(57) Abstract

This invention relates to the area of systemic, tissue–specific non–viral gene delivery. The present invention provides a novel method to prepare ligand–directed, PEG–stabilized complex as gene delivery systems for targeted gene therapy. Due to the presence of PEG these novel complexes have longer circulating times than conventional ligand–liposome complexes. In addition, due to the presence of the ligand in the complex with PEG, these complexes are tissue targeting. Their small size further makes them very desirable for *in vivo* use.

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TITLE OF THE INVENTION

LIGAND-PEG POST-COATING STABILIZED LIPOPLEX AND POLYPLEX FOR TARGETED GENE DELIVERY

FIELD OF THE INVENTION

This invention relates to the methods to prepare ligand-PEG post-coating stabilized liposome-DNA complex (Lipoplex) or polymer-DNA complex (Polyplex) for targeted gene delivery *in vivo*. The complexes prepared by ligand-PEG post-coating after complexing with DNA have a defined size with the ligand-PEG coating outside and have the capability of targeted gene delivery with systemic administration.

BACKGROUND OF THE INVENTION

Due to the rapid advancement of genetics, gene therapy is becoming a promising strategy for the treatment of cancer and other diseases (1-3). Current gene therapy approaches employ either viral or non-viral vector systems (4, 5). The limitations of a viral approach are related to their lack of targeting and to residual viral elements that can be immunogenic, cytopathic, or recombinogenic (5). Non-viral gene transfer vectors could circumvent some of the problems associated with using viral vectors. Progress has been made toward developing non-viral, pharmaceutical formulations of genes for *in vivo* human therapy, particularly cationic liposome-mediated gene transfer systems (4-8). Cationic liposomes are composed of positively charged lipid bilayers and can be complexed to negatively charged, naked DNA by simple mixing of lipids and DNA such that the resulting complex has a net positive charge. The liposome-DNA complex (lipoplex) is easily bound and taken up by cells with a relatively high transfection efficiency (8). Features of cationic liposomes that make them versatile and attractive for DNA delivery include: simplicity of preparation; the ability to complex large amounts of DNA; versatility in use with any type and size of DNA or RNA; the ability to transfect many different types of cells, including non-dividing cells; and lack of immunogenicity or biohazardous activity (reviewed in 4, 9). Notably, the introduction of wtp53 by a liposome-DNA complex partially inhibited the growth of mammary (10) or glioblastoma (11) tumors in nude mice. More importantly from the perspective of human cancer therapy, cationic liposomes have been proven to be safe and efficient for *in vivo* gene delivery (8, 12). More than twenty clinical trials are now

underway using cationic liposomes for gene delivery, and liposomes for delivery of small molecule therapeutics (e.g., chemotherapeutic and antifungal agents) are already on the market (2).

A. Tumor Targeted Liposome Delivery

One disadvantage of cationic liposomes is that they lack tumor specificity and have relatively low transfection efficiencies as compared to viral vectors. However, this can be dramatically increased when the liposomes bear a ligand recognized by a cell surface receptor. Receptor-mediated endocytosis represents a highly efficient internalization pathway in eukaryotic cells (13-15). The presence of a ligand on a liposome facilitates the entry of DNA into cells through initial binding of ligand by its receptor on the cell surface followed by internalization of the bound complex. Once internalized, sufficient DNA escapes the endocytic pathway to be expressed in the cell nucleus. A variety of ligands have been examined for their liposome-targeting ability, including folic acid, a vitamin necessary for DNA synthesis (13, 16). Folate receptor levels are found elevated in various types of cancer including breast cancer (17-22), and most significantly, the folate receptor recycles during the internalization of folate in rapidly dividing cells such as cancer cells (23). Folate-conjugated macromolecules and liposomes have also been shown to be specifically taken up *in vitro* by receptor-bearing tumor cells (24, 25). Thus the folate receptor is considered to be useful as a prognostic tumor marker for cancer and as a potential target for drug delivery in the therapy of malignant cell growth (16, 22, 24, 26).

Recently, we have developed a folate-liposome-DNA system for systemic wtp53 gene therapy of SCCHN and examined its ability to sensitize SCCHN to radiation *in vitro* and *in vivo* (27). These experiments demonstrated the tumor selectivity and high *in vivo* transfection efficiency of this systemically delivered liposome complex, and that the combination of intravenous folate-liposome-p53 and radiation was able to eliminate and prevent the recurrence of established SCCHN xenograft tumors for extended periods of time. Interestingly, this folate-liposome-DNA system can also target to human breast cancer *in vitro* and *in vivo* (27).

The limitations of the above ligand-liposome-DNA complex or ligand-lipoplex that we encountered are low stability (must be freshly prepared before use) and high *in vivo* clearance (low serum half-life). Thus, only a limited amount of i.v. injected lipoplex actually reaches the tumor. These two areas must be improved upon for continued pharmaceutical development of the folate lipoplex. One way to overcome these limitations is to coat the lipoplex with inert

polymers to build up a sterically repulsive shield that protects the complex from nonspecific opsonization by plasma proteins, thus interfering with liposome recognition and clearance from circulation by the macrophages of the reticuloendothelial system (RES) (28, 29).

B. Sterically Stabilized Liposomes

Numerous studies have reported that modifications of unliganded liposomes with polymers such as polyethylene glycol (PEG) prolong the length of time in blood circulation as a result of a reduced rate and extent of uptake by the RES (reviewed in 28). These liposomes have been characterized as "sterically stabilized" liposomes or "Stealth™" liposomes, on the basis of their enhanced *in vivo* stability and reduced reactivity to plasma proteins (28). Hong K, et al. (30) produced a stable cationic liposome-DNA complex by including a small amount of PEG-phospholipid conjugate in the formulation. The preparation was reported to be stable for months at 4°C and gave reproducible high transfection activity in mouse lungs after intravenous injection. Recently, Cullis' group (31, 32) described a detergent dialysis procedure to prepare a PEG-lipoplex, which allows encapsulation of plasmid DNA within a lipid envelope, where the resulting particle is stabilized by an existing PEG coating. However, the transfection activity of this PEG-lipoplex relied upon the dissociation of the PEG coating, transforming the complex from a stable particle to a transfection-competent one. Although this procedure works *in vitro*, the requirement of PEG-coating dissociation severely limits its ability to systemically deliver genes *in vivo*. It is difficult to control the timing of the PEG-coating dissociation after i.v. injection of the particle, circulation in the blood stream and passage through the blood vessel wall, to reach the target site. As for ligand-targeted liposome-DNA complexes, there is less data in the literature with regards to PEG-stabilization. We prepared cationic folate-PEG-liposomes to complex DNA, using a modification of the methods reported by Dr. Low's group (24, 33). Using these methods, we encountered a problem in that the prepared cationic PEG-liposomes had a PEG-coating layer on their outer surface, which interfered with their subsequent complexing and condensing with DNA, a key step in forming lipoplexes. Therefore, a higher lipid/DNA ratio was required. The result was that the complexing was neither efficient nor complete, and the size of the complexes was too large (300-1000 nm) for the *in vivo* systemic gene delivery to be feasible. A report by Zalipsky et al. (72) uses a different chemistry and a different methodology than the one described herein to attach a ligand-PEG-lipid conjugate to a liposome. Their

methodology employs a lipid anchor (DSPE) to attach a ligand to a liposome vesicle via a PEG linking moiety.

C. Polyethylenimine – the versatile cationic polymer for gene delivery

Polyethylenimine (PEI) is the organic macromolecule with the highest cationic-charge-density potential, and a versatile vector for gene and oligonucleotide transfer *in vitro* and *in vivo*, as first reported by Boussif et al. (66). Since then, there has been a flurry of research aimed at this polycation and its role in gene therapy (73). Cell-binding ligands can be introduced to the polycation to 1) target specific cell types and 2) enhance intracellular uptake after binding the target cell (13). Erbacher et al. (67) conjugated the integrin-binding peptide 9-mer RGD via a disulfide bridge and showed physical properties of interest for systemic gene delivery. Ogris et al. (68) conjugated transferrin to PEI 800 kDa and compared the *in vitro* and *in vivo* transfection activities of the DNA/transferrin-PEI complex before and after PEGylation (i.e., covalent coupling of PEG). PEGylation of the complexes strongly reduced plasma protein binding and erythrocyte aggregation, their size was stabilized and the surface charge was reduced. The PEGylation prolonged the circulation of the complex in the blood after i.v. injection. Targeted gene delivery into subcutaneously growing tumors after systemic application was achieved using this PEGylation sterically stabilized DNA/transferrin-PEI complex, whereas non-PEGylated complexes gave predominant gene expression in the lungs associated with considerable toxicity (69). This strategy differs from the PEG post-coating method in that the ligand transferrin is already conjugated to the PEI prior to addition of PEG. Although the results are encouraging, the major shortcoming of the strategy is that the ligand transferrin lies inside the PEG shield and thus limits its accessibility to the corresponding receptor on target cells. Another drawback is that the DNA/transferrin-PEI was PEGylated with a succinimidyl derivative of PEG propionic acid (M-PEG-SPA), which reacts randomly with primary amino groups present on both the PEI and the transferrin molecules (69). Therefore, the ligand transferrin itself can be reacted by M-PEG-SPA interfering with or blocking binding to its receptor, or even completely inactivating the ligand.

The above problems can be avoided by using the "ligand-PEG post-coating" method disclosed in the present invention, while preserving all the advantages of PEGylation.

SUMMARY OF THE INVENTION

The present invention discloses a novel strategy for preparing the ligand-PEG-liposome-DNA or ligand-PEG-PEI-DNA complexes. This is referred to as a ligand-PEG post-coating method. With this strategy, ligand-PEG is linked after the DNA has been condensed inside the liposomes (or PEI). Hence the PEG layer will coat only the outside of the DNA-liposome complex (lipoplex) or DNA-PEI complex (polyplex), and will not interfere with the internal structure of the complexes. Therefore, the complex has a condensed DNA-cationic lipid or PEI structure inside and a PEG coating outside, with ligand at the distal end of the coating PEG. We designate this strategy a "ligand-PEG post-coating" method to distinguish it from previous methods (24, 29, 33, 68, 72).

The ligand-PEG post-coating method takes advantage of the protective and long-circulating properties of the reported PEG-liposomes such as "sterically stabilized" liposomes or "Stealth™" liposomes, while keeping the unique characteristics of ligand-cationic liposome-DNA complexes. The ligand-PEG post-coated lipoplexes have low cytotoxicity and the capability of SCCNH tumor-targeted gene delivery after systemic administration. This strategy is a very useful and promising way to design and develop a targeted gene delivery system for systemic gene therapy.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the scheme for preparation of a folate-liposome-DNA complex by a "Post-coating Method". Note that before folate-PEG-SH, the solid lines show the SPDP method and the dashed lines show the 2-iminothiolane method.

Figure 2 shows the folate-PEG-liposome-mediated transfection of JSQ-3 cells and the effect of serum on their transfection. The LipA-PEG-F complexed with plasmid pSVb DNA (27) was prepared by the "Post-coating Method". LipA consists of equal molar DOTAP and DOPE plus 5% molar DOPE-MB. LipA-PEG complexed with pSVb was prepared similarly by post-coating PEG without folate. JSQ-3 cells in a 96-well plate ($1x10^4$ cells/well) were transfected by 1 μg DNA/well of LipA-PEG-F(pSVb) or LipA-PEG(pSVb) with or without 10% fetal bovine serum. 2 days later, the cells were lysed and β-galactosidase expression was measured by colorimetric assay. Means of duplicates were plotted.

Figure 3 shows the specificity of folate-PEG-liposome-mediated gene transfection of JSQ-3 cells. LipA-PEG1-F and LipA-PEG2-F were prepared by the "Post-coating Method", SPDP method and 2-iminothiolane method, respectively . JSQ-3 cells in A 96-well plate ($1x10^4$ cells/well) were transfected with serially diluted LipA-PEG1-F(pSVb) or LipA-PEG2-F(pSVb), starting from 0.32 μg DNA/well. For ligand competition, free folic acid (FA) was added just before transfection to a final concentration of 1 mM. 2 days later, the cells were lysed and β-galactosidase expression was measured by colorimetric assay. Means of duplicates were plotted.

Figures 4A-I show the results of "post-coated" folate-PEG-liposome-mediated systemic gene transfection *in vivo*. LipA-PEG1-F was prepared by the SPDP method and LipA-PEG2-F by the 2-iminothiolane method.

Figures 5A-D show spectrophotograms of purified F-PEG-PDP and Gal-PEG-PDP, before and after the addition of DTT, using a Beckman DU640 spectrophotometer from 200 nm to 700 nm.

DETAILED DESCRIPTION OF THE INVENTION

The ligand-PEG post-coating method takes advantage of the protective and long-circulating properties of the reported PEG-liposomes such as "sterically stabilized" liposomes or "Stealth™" liposomes, while keeping the unique characteristics of ligand-cationic liposome-DNA complexes. The ligand-PEG post-coated lipoplexes have low cytotoxicity and the capability of SCCNH tumor-targeted gene delivery after systemic administration. This strategy is a very useful and promising way to design and develop a targeted gene delivery system for systemic gene therapy.

It is important to note that the methodology described herein does not employ a lipid anchor, nor is PEG used as a "linking moiety". Moreover, it should be noted that the liposome used by Zalipsky et al. (72) is an empty vesicle, not one encapsulating DNA. Most significantly, the conjugate described by Zalipsky et al. (72) would not work to deliver the DNA-liposome complex produced by our method which has demonstrated small size and demonstrated efficacy. Attachment of the DSPE-lipid anchor to this liposome-DNA complex would effectively destroy its structure and therefore its potential as a delivery system for gene therapy.

Folate is used as an example of a ligand for lipoplex formation, as illustrated in Figure 1 which depicts a scheme for preparation of folate-PEG liposome-DNA complexes. First, DNA

is complexed and condensed with cationic liposomes, containing 1-10%, preferably 5%, molar of DOPE-Maleimidophenyl butyrate (DOPE-MPB), or any other sulfhydryl-reacting molecule-lipid conjugate, in their formulation. The sulfhydryl group is necessary for subsequent conjugation. A dithiopyridine (PDP) group is first introduced at one end of PEG-bis-amine and then folate is introduced at the other end. The folate-PEG-PDP is then reduced by dithiothreitol (DTT), or any other reducing reagent, to produce free sulfhydryl group, folate-PEG-SH. This complex is then reacted with the maleimide group of MPB, or any other sulfhydryl-reacting molecule, on the DNA-liposomes to link the folate-PEG to the DNA-liposomes (Fig.1, solid line flowchart). This method is called the SPDP method although other amine-reacting crosslinkers can also be used. An alternative method of the strategy is shown in Fig.1, dashed line, wherein the folate is first linked to one end of PEG-bis-amine by reacting it with folate-NHS. A free sulfhydryl (-SH) group is then introduced directly to the other end by reacting it with 2-iminothiolane. The resulting folate-PEG-SH is then post-coated onto DNA-liposomes. This method is called the 2-iminothiolane method, although other thiol-introducing reagents can also be used. The advantage of the 2-iminothiolane method is that the whole process can be carried out in aqueous phase, which is especially suitable for ligands unstable in organic phase, e.g., peptide or protein ligands such as RGD, Fas-ligands, EGF, FGF, antibodies or their fragments, etc.

Cationic polymers, e.g., polylysine, protamine or polyethylenimine can also be used to replace the cationic liposome. PEI is used as an example, similar to that of liposomes. The maleimide groups of MB, or other sulfhydryl-reacting molecules, are introduced to PEI, in the range of 0.1-25% modification of amines in the PEI molecule. DNA is complexed and condensed first with PEI-MB, at a N/P ratio (amine nitrogen of PEI vs. phosphate of DNA, molar ratio) of 1-50, preferably 5-15, as described (66, 67). After addition of HEPES buffer to a final concentration of 10-20 mM, to adjust pH to 7.4-8.0, the freshly reduced ligand-PEG-SH is added to the polyplex and reacted for 2-6 hours at room temperature, or overnight at 4°C. The post-coated polyplex can be used directly, or purified by Sepharose CL-4B chromatography to remove the unconjugated ligand-PEG. A galactose-PEG-SH post-coated PEI-DNA complex prepared as described above and detailed below in Example 8 showed enhanced stability, increased resistance to serum, and reduced toxicity both *in vitro* and *in vivo*. The intravenous injection of the galactose-PEG post-coated polyplex resulted in significantly increased reporter gene

expression in mouse liver and much reduced expression in mouse lung, whereas the non-PEGylated polyplex gave predominant gene expression in mouse lung and was associated with considerable toxicity.

Other conjugation strategies can also be used to link a ligand at one end of PEG and an active group, e.g., a thiol group (-SH), at the other end for the subsequent "post-coating". Alternatively, an active group (with protection) can be linked first, followed by a ligand at the other end. After deprotection (like the reduction step in the SPDP method), the free active group is ready for "post-coating". An alternative strategy is to make ligand-PEG-succinimidyl (active) ester, then react this with the primary amino groups in lipoplex (from DOPE) or polyplex (from PEI) for post-coating. In this case, no prior modification of lipids or PEI to introduce a reactive group (e.g., maleimide group of MB) is required. The chemical or biochemical procedures involved in the invention are common techniques familiar to those skilled in the art.

Since ligand-PEG is linked after the DNA has been condensed inside the liposomes, the PEG layer will coat the outside of the DNA-liposome complex and will not interfere with the internal structure of the DNA-liposome complex. Therefore, the complex has a condensed DNA-cationic lipid structure or acentric onion-like core structure (see Example 6 below, detailing an electron microscopy analysis) inside and a PEG coating outside, with folate at the distal end of PEG.

The ligand-PEG "post-coating" method takes advantage of the protective and long-circulating properties of the reported PEG-liposomes such as "sterically stabilized" liposomes or "Stealth™" liposomes, while keeping the unique characteristics of ligand-cationic liposome-DNA complexes. The ligand-PEG post-coated liposomes have the capability of tumor-targeted gene delivery after systemic administration and are suitable for systemic gene therapy *in vivo*.

The invention is not limited to the use of any specific targeting ligand. Folate is used as an example of a targeting ligand. Other ligands can easily be used in the invention with minor modifications. The ligand can be any ligand the receptor for which is differentially expressed on the target cell. Examples include other vitamins, EGF, insulin, FGF, Heregulin, RGD peptides or other polypeptides reactive to integrin receptors, antibodies or their fragments. In a preferred embodiment the ligand is folate. For ligands unstable in organic phase, e.g., polypeptide or protein ligands such as EGF, FGF, antibodies or their fragments, etc., the reactions to make the

ligand-PEG-SH can be carried out in aqueous phase with minor modifications familiar to those skilled in the art.

The invention is not limited to the use of any specific coating polymer for "post-coating". The coating polymer can be any polymer with inert chemical activity within the polymer chain and active groups at the two ends for conjugation. Zalipsky S et al., US Patent No. 5,395,619 (74) disclosed, other than PEG, a series of polymers capable of forming long-circulating liposomes. In the preferred embodiment the polymer is PEG with primary amine groups at the two ends as active groups.

The invention is not limited to the delivery of any specific type of DNA. Any polynucleotides that can be complexed by cationic liposomes or polymers can be used in the invention. Examples of the polynucleotides include but are not limited to: plasmid DNA, DNA fragments, oligonucleotides, oligodeoxynucleotides, chimeric RNA/DNA oligonucleotides, RNA, ribozymes, etc. Anionic peptides, polymers, synthetic or natural molecules can also be used in the invention so long as they can be complexed by cationic liposomes or polymers. Other examples of molecules which can be delivered via ligand-PEG post coated cationic liposomes include a gene, high molecular weight DNA, plasmid DNA, an antisense oligonucleotide, peptide nucleic acids, a chemical agent such as a chemotherapeutic molecule or any large molecule including, but not limited to DNA, RNA, viral particles, growth factors, cytokines, immunomodulating agents and other proteins, including proteins which when expressed present an antigen which stimulates or suppresses the immune system.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art, that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the disclosure.

Example 1

Preparation of ligand-PEG-SH for post-coating by SPDP method

This example discloses the SPDP reaction procedure for preparation of ligand-PEG-SH for post-coating. This is illustrated in Figure 1 by the solid line flowchart.

A. Preparation of PDP-PEG-NH2

30 µmol Polyoxyethylene bis(amine) (NH2-PEG-NH2) (M.W. 3350, Sigma) in 2 ml dry chloroform was stirred at room temperature while a solution of 30 µmol *N*-succinimido-3-(2-pyridyldithio)propionate (SPDP) (Sigma) in 1 ml dry chloroform was added dropwise over a period of 10-15 minutes. 10 µl TEA was added to the solution and stirred for an additional 15-30 minutes. This solution was purified to remove PDP-PEG-PDP or unreacted NH2-PEG-NH2 as described (48). The product was confirmed by thin layer chromatography (TLC) (chloroform/methanol/acetic acid 100/30/2) as described by Haselgrubler et al. (48).

B. Preparation of Folate-PEG-PDP

N-Hydroxysuccinimide ester of folic acid (folate-NHS) was prepared by reacting 1 gram folic acid (F, Sigma), in 30 ml dry dimethyl sulfoxide plus 0.5 ml triethylamine (TEA, Sigma), with 0.52 g N-hydroxysuccinimide (NHS, Sigma) in the presence of 0.94 g dicyclohexylcarbodiimide (DCC, Fluka) overnight at room temperature (75). The solution was filtered and purified as described by Lee RJ and Low PS (75). PDP-PEG-NH2 was reacted with 2-5 molar excess of folate-NHS in dry chloroform plus 2 molar of TEA overnight at room temperature. One volume of chloroform was added to the solution then washed with PBS 4-6 times, centrifuging at 1000-2000 rpm for 15 minutes between each wash. The clear yellow chloroform solution was evaporated to dryness to obtain the yellow powder product, folate-PEG-PDP. The product was confirmed by TLC (chloroform/methanol/acetic acid 100/30/2).

C. Reduction of PDP to obtain folate-PEG–SH

Folate-PEG-PDP was dissolved in 10 mM HEPES buffer, pH 7.4. Freshly prepared 500 mM dithiothreitol (DTT, Sigma) in water was added to reach 50 mM final concentration. The solution was stirred for 30 minutes at room temperature and then desalted by column chromatography (10DG, BioRad) or by Centricon 3 ultrafiltration to obtain reduced folate-PEG–SH. The free –SH group is not stable and will oxidize easily. Therefore, the reduction should be done immediately before "post-coating" and folate-PEG–SH should be used within 1-2 hours after desalting. EDTA may be added to a 1 mM final concentration to help stabilize the

folate-PEG–SH. The purified folate-PEG–SH can be frozen at –20°C under inert gas for later use. The free –SH group was confirmed by DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) titration (76). Folate was measured by absorption at 363 nm. The molar ratio of folate/–SH in the purified product was approximately 0.9-1.1.

Example 2

Preparation of ligand-PEG-SH for post-coating by direct thiolation (2-iminothiolane method)

This example discloses the reaction procedure of preparing ligand-PEG-SH for post-coating by direct thiolation. This is illustrated in Figure 1 by the dashed line flowchart.

Folate is linked to one end of PEG-bis(amine) first. 30 µmol Polyoxyethylene bis(amine) (NH2-PEG-NH2) in 2 ml dry chloroform was mixed with 30 µmol N-Hydroxysuccinimide ester of folic acid (folate-NHS) in 1 ml dry chloroform. 10 µl TEA was added to the reaction solution and the mixture stirred for 0.5-2 hours at room temperature. One volume of chloroform was added to the solution, and then washed with PBS 4-6 times, centrifuging at 1000-2000 rpm for 15 minutes between each wash. The clear yellow chloroform solution was evaporated to dryness. The product was purified to remove folate-PEG-folate or unreacted NH2-PEG-NH2 as described (48). The product was confirmed by thin layer chromatography (TLC) (chloroform/methanol/ acetic acid 100/30/2) with iodine staining.

Free -SH is introduced to the other end of folate-PEG directly by reacting with 2-iminothiolane. The reaction is performed in aqueous solution. Folate-PEG-NH2 was dissolved in 100 mM HEPES, pH 8.0. 2-5 molar excess of 2-iminothiolane HCl (Sigma) in PBS was added and stirred for 0.5-2 hours at room temperature. The resulting folate-PEG-SH was purified by desalting column chromatography (10DG, BioRad) or by Centricon 3 ultrafiltration to obtain reduced folate-PEG–SH. The free –SH group was confirmed by DTNB titration and folate was measured by absorption at 363 nm. The molar ratio of folate/–SH in the purified product was approximately 0.9-1.1.

Example 3

"Post-coating" of cationic liposome-DNA complex (Lipoplex)

This example discloses the procedure of ligand-PEG-SH post-coating onto DNA-liposomes.

A. Preparation of cationic liposomes

DOPE-MPB was prepared by reacting DOPE with succinimidyl-4-(*p*-maleimidophenyl)butyrate (SMPB, Sigma) in dry chloroform in the presence of TEA (65). The maleimide group of MPB is reactive to the sulfhydryl group and serves as the conjugating molecule on the liposomal surface. Other thiol-reactive groups can also be used here, such as other maleimido-containing molecules, etc. DOPE-MPB is also available from Avanti Polar Lipids, Inc., Alabaster, AL.

Cationic liposomes LipA were prepared as follows: a chloroform solution of 5 μmol dioleoyltrimethylammonium-propane (DOTAP), 5 μmol DOPE (Avanti Polar Lipids, Inc., Alabaster, AL) and 0.1-1 μmol DOPE-MPB were mixed together in a round-bottom flask, and the chloroform was evaporated under reduced pressure. 10 ml pure water, was added to the flask to suspend the lipids, then sonicated for 10 minutes in a bath-type sonicator at 4°C. The final concentration of the liposome was 1-2 nmol/μl total lipids. Other cationic liposomes were also prepared in similar fashion with the compositions shown below:

Table 1

Composition of cationic liposomes for post-coating

Liposomes	Composition	Molar ratio	Preferred ratio
LipA	DOTAP/DOPE/DOPE-MPB	5/5/(0.1-1)	5/5/0.5
LipB	DDAB/DOPE/DOPE-MPB	5/5/(0.1-1)	5/5/0.5
LipD	DOTAP/Chol/DOPE-MPB	5/5/(0.1-1)	5/5/0.5
LipE	DDAB/Chol/DOPE-MPB	5/5/(0.1-1)	5/5/0.5

LipD and LipE were sonicated at 65°C instead of 4°C.

Other liposome preparation methods can also be used to prepare the cationic liposomes. For example, the ethanol injection method modified from that described by Campbell (80) was used successfully in the present invention. In brief, all lipids were solubilized in ethanol and mixed, injected into vortexing pure water of 50-60°C with a Hamilton syringe. The solution was vortexed for a further 10-15 minutes. The final concentration was 1-2 μM total lipids. The ethanol injection method is faster, easier and more robust.

Since we have found that the maleimide group is not stable in aqueous solution with pH >7, the liposomes should be prepared in water (pH 5-6.5). The pH can be adjusted to 7.5-8.0 before "post-coating" with 1 M HEPES buffer, pH 7.5-8.0, to facilitate the post-coating reaction.

B. "Post-coating" of DNA-liposomes complex

Plasmid DNA was complexed with MPB-liposomes in water or 10 mM HEPES buffer, pH 7.0, as described (34) at DNA/lipid ratios 1/6-1/26 (μg/nmol), preferably 1/10-1/20. Before "post-coating", 1 M HEPES buffer, pH 7.5-8.0, was added to the MPB-liposome-DNA complexes to a final concentration of 10-20 mM. Folate-PEG-SH was added at 0.5-5 molar excess to MPB-DOPE, preferably 1-2 molar excess and stirred several hours to overnight at room temperature in the dark. The resulting folate-PEG-LipA-DNA is used directly with 5% dextrose for *in vivo* gene transfection with pSVb as a reporter gene. For *in vitro* experiments, we tested the folate-PEG-Lip-DNA solution for transfection without further purification or after purification by Sepharose CL-4B chromatography to eliminate the unlinked folate-PEG-SH. Sepharose CL-4B chromatography confirmed that more than 50% of ligand-PEG-SH was linked to the lipoplex.

Example 4

In vitro gene transfection by "post-coated" folate-PEG-Lip-pSVb

This example describes the *in vitro* gene transfection efficiencies of "post-coated" folate-PEG-liposomes using a reporter gene.

The medium used here was folate-free medium (RPMI-1640 folate-free, Gibco). 1 x 10^4 cells were plated in each well of a 96-well plate or 5 x 10^4 cells/well in a 24-well plate. 24 hours later, the cells were washed once with medium without serum or antibiotics and 100 μl transfection solution containing various amounts of folate-PEG-Lip-pSVb or PEG-Lip-pSVb as well as pSVb alone were added to each well. After 5 hours of transfection at 37°C, an equal amount of medium containing 20% fetal bovine serum were added to each well. 48 hours later, the cells were washed once with PBS and lysed in 1X reporter lysis buffer (Promega). The cell lysates were treated with 100 μl 150 μM O-nitrophenyl-β-galactopyranoside in 20 mM Tris (pH 7.5) containing 1 mM $MgCl_2$ and 450 mM β-mercaptoethanol at 37°C for 0.5 hour. The reaction was stopped by the addition of 150 μl/well of 1 M Na_2CO_3. The absorbance was determined at 405 nm. Purified β-galactosidase (Boehringer) was used as a standard. The results were

expressed as milliUnits of β-galactosidase equivalent per mg of total protein. For histochemical studies of ligand-liposome-pCMVb transfection, 60% confluent cells in 24-well plates were transfected for 5 hours as described above. After an additional 2 days in culture, the cells were fixed and stained with X-gal. Transfection efficiency was calculated as the percentage of blue-stained cells.

Figure 2 shows the *in vitro* folate-PEG-liposome-mediated transfection of JSQ-3 cells and the effect of serum on the transfection efficiency. PEG "post-coated" liposomes show resistance to serum and folate-PEG-LipA resulted in two-fold higher reporter gene expression than LipA-PEG without folate ligand.

Figure 3 shows the specificity of folate-PEG-liposome transfection. Free folic acid can block the transfection activity of folate-PEG-liposomes to the level of the LipA-PEG, demonstrating that the transfection of the folate-PEG-liposomes is mediated by the folate through the folate-receptor.

Example 5

In Vivo Gene Transfection in Nude Mouse Tumor Model by i.v. Injection of the "post-coated" folate-PEG-Lip-pSVb

This example demonstrates the ability of the "post-coated" folate-PEG-Lip-pSVb to selectively target tumor tissue *in vivo* after systemic administration.

NIH3T3 cells transformed with a human Ha-Ras gene were subcutaneously injected in the flank of 4-6 week old female nude (NCr nu-nu) mice. The tumors were allowed to develop to a size of 100 mm^3. The "post-coated" folate-PEG-Lip-pSVb were prepared as described in Examples 1-3, wherein LipA-PEG1-F indicates the "post-coated" folate-PEG-Lip-pSVb prepared by SPDP method (Examples 1 and 3) and LipA-PEG2-F indicates that prepared by the direct thiolation method (Examples 2 and 3). LipA-PEG1-F, LipA-PEG2-F or pSVb plasmid alone (in 5% dextrose) was injected intravenously via the tail vein, at 50 μg of plasmid DNA/300 μl/animal. Three days and 10 days after DNA injection, the tumors as well as mouse organs were excised, cut into 1 mm sections, washed once with PBS, and fixed with 2% Formaldehyde-0.2% Glutaraldehyde for 4 hours at room temperature. The fixed tumor sections were washed 4 times, each for 1 hour, and stained with X-Gal solution plus 0.1% NP-40 (pH 8.5) at 37°C overnight. The stained tumor sections were embedded and sectioned using normal histological procedures

and counter-stained with nuclear fast red. Four sections per tumor were examined to evaluate the β-galactosidase gene expression, as indicated by the blue stained cells.

Figures 4A-I show the "post-coated" folate-PEG-liposome-mediated systemic gene transfection *in vivo*. As shown in Figures 4A and 4B, 3 days after the i.v. injection of LipA-PEG-F complexed with pSVb, 30-60% of tumor cells in nude mouse xenograft stained blue, indicating the tumor delivery and expression of reporter gene β-galactosidase. The normal mouse tissues, including lung and liver, were not stained blue, except a few macrophages (Figures 4G and 4H), demonstrating that the PEG "post-coated" liposomes can deliver the complexed gene selectively to tumors *in vivo*. Although the folate-liposome without PEG coating showed 30-40% tumor cell blue-staining (Figure 4C), the gene expression disappeared within 6-10 days (Figure 4F). But the PEG "post-coated" liposomes injected groups still showed some (10-40%) blue-staining in the tumors after 10 days, demonstrating that the ligand-PEG "post-coated" liposomes can last longer after systemic injection and give sustained gene expression in the tumors. Plasmid pSVb only injected group showed no tumor cells staining blue (Figure 4I).

The ligand-PEG "post-coated" liposomes can deliver the complexed genes selectively to the tumor *in vivo* after systemic administration, and give sustained gene expression in the tumor, with an *in vivo* transfection efficiency of 30-60%. Therefore, this system is very useful and promising for *in vivo* gene delivery and gene therapy.

Example 6

Electron microscopic analysis of the ligand-PEG "post-coated" cationic liposomes

Liposomes can be observed with Electron Microscopy (EM), either Transmission Electron Microscopy (TEM) with negative staining or Scanning Electron Microscopy (SEM). EM can reveal the structure and size distribution of liposomes. EM can also be used for quality control of liposomal preparation. We observed the ligand-cationic liposomes under Transmission Electron Microscope with negative staining.

The copper grid with Formvar and Carbon coating (Electron Microscopy Sciences, Fort Washington, PA) was used in the study. Ligand-PEG "post-coated" cationic liposome-pSVb complexes were prepared as described in Examples 1-3. One drop of the liposome complex was added to the grid. 5 minutes later, excess liquid was removed by attaching a filter paper to the edge of the grid. One drop of 4% Uranium Acetate was then added onto the grid for negative

staining. 5 minutes later, excess liquid was also removed by attaching a filter paper to the edge of the grid. The grid was air dried at room temperature for 15 minutes before being put into the sample chamber of the TEM. The TEM JEOL-1200EX was used in the study according to the manufacturer's instruction. Photos were taken at magnitudes of 10-50k, 60 kVolt. The liposome samples on the grid were prepared and stained freshly and observed within one hour.

Many publications indicate that cationic liposome-DNA complexes have a diverse structure and size ranging from 100 nm to 1000 nm. In our study, we observed unexpectedly that the ligand-liposome-DNA complexes disclosed in the invention have much smaller size and much more even size distribution. Cationic liposome LipA itself has a size of 25-50 nm, average 35 nm (in diameter). When DNA was complexed with LipA, an interesting "irregular or acentric onion-like core structure" in the core of the LipA-DNA complex was observed, with evenly distributed sizes of 35-65 nm (50 nm in average). After the ligand-PEG "post-coating" process, the size of liposomes increased only slightly, becoming 35-80 nm (60 nm in average), indicating a 10-15 nm thick layer of folate-PEG was "post-coated" on the LipA-DNA complex.

To reach target tumor *in vivo*, the liposomes must be first resistant to serum and then pass through the blood vessel (capillary) wall. The ligand-PEG "post-coated" liposomes disclosed in the invention can meet these two requirements. The ligand-PEG-Lip-DNA is very resistant to serum as shown in Example 4. Using fluorescence labeling and colloidal gold labeling with silver enhancement, it has been demonstrated that 80-100 nm-size PEG-stabilized liposomes could penetrate into the extravascular, interstitial space among tumor calls and scatter throughout the entire tumor region, in several solid tumor models with i.v. injection of the liposomes (77-79). Therefore, the ligand-PEG "post-coated" liposome-DNA complexes of 35-80 nm size disclosed in the invention could pass through the capillary wall to reach the target, as confirmed by the *in vivo* gene transfection data disclosed in Example 5.

Example 7

Stability assay of the ligand-PEG "post-coated" liposomes

Stability is a critical issue for liposomal pharmaceuticals. Liposome solutions should be stable for an extended period of time after preparation to allow for shipment and storage, without significant loss of their biological/pharmaceutical activities, to be useful as therapeutic agents. In light of the future clinical use of the ligand-liposome-therapeutic molecule complex of this

invention, in this example we examined the stability of the ligand-liposomes and the ligand-liposome-DNA complexes. The ligand-PEG "post-coated" liposome-pSVb complexes were stored under nitrogen in the dark at 4°C for various periods of time, up to 12 months. On the day of the assay, the stored liposomes, as well as freshly prepared liposomes, were used to transfect JSQ-3 cells using the transfection assay as described in Example 4.

LipA-DNA or folate-LipA-DNA complexes without PEG coating lost their transfecting activity in 1-3 days. When ligand-PEG was "post-coated", the liposomes became stabilized and retained transfecting activity for a prolonged period of time. 100% of their activity was evident for one month and 50-60% transfecting activity still remained after 6 months in storage. Therefore, the ligand-PEG "post-coated" cationic liposomes disclosed in the invention are more stable and have a longer shelf life, which are important considerations for practical use.

Example 8

Preparation of Galactose-PEG post-coated polyethylenimine-DNA complex (polyplex)

This example discloses a procedure to prepare a galactose-PEG post-coated PEI-DNA complex. Galactose or galactosylated ligands have high affinity to asialoglycoprotein receptor which is present at high density on hepatocytes and hepatomas (13). Galactose-PEG post-coated PEI-DNA complex will be useful for targeted gene delivery to liver for gene therapy of liver diseases.

A. Modification of PEIs

Branched PEI with average molecular weight of 25 kDa (designated P25) was purchased from Aldrich. Linear PEI with average molecular weight of 25 kDa (designated LP25) was purchased from Polysciences, Inc. Both PEIs were reacted with maleimide-containing active ester to introduce maleimide group, similar to that of DOPE-MB in Example 3. The followings are examples of (but not limited to) the maleimide-containing active esters that can be used: 3-Maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) (Sigma M2786), Succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB) (Sigma M6286) and Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (Sigma M5525). 0.1-0.2 g P25 in 3 ml dry chloroform (LP25 in 2 ml chloroform and 1 ml DMSO) was added to 50 mg MBS/1 ml DMSO and 100 µl triethylamine (Sigma). The mixture was stirred overnight at room temperature. 10 ml pure water was added, vortexed and centrifuged (5000 rpm, 10 minutes).

The modified PEI, P25-Maleimidobenzoyl (MB), was solubilized in the aqueous phase, while LP25-MB was a white or light yellow solid between the two phases. The P25-MB was purified by a 10-DG desalting column (BioRad) equilibrated with water. The solid LP25-MB was taken out, washed twice with water, dried and weighed. The solid LP25-MB was solubilized in water by adding 1 N HCl dropwise until totally clear.

B. Preparation of Galactose-PEG-PDP

This procedure is similar to that of F-PEG-PDP in Example 1. 30 μmol Polyoxyethylene bis(amine) (NH2-PEG-NH2) (M.W. 3350, Sigma) in 2 ml dry chloroform was stirred at room temperature. A solution of 30 μmol *N*-succinimido-3-(2-pyridyldithio)propionate (SPDP) (Sigma) in 1 ml dry chloroform was added dropwise over a period of 10-15 minutes. 10 μl TEA was added to the solution and stirred for an additional 15-30 minutes. The obtained PDP-PEG-NH2 was then reacted with 1-3 molar excess of D-galactopyranosylphenyl isothiocyanate (Sigma) in dry chloroform plus 2-6 molar of TEA overnight at room temperature. One volume of chloroform was added to the solution then washed with PBS 4-6 times, centrifuging at 1000-2000 rpm 15 minutes between each wash. The clear chloroform solution was evaporated to dryness to obtain the product, Galactose-PEG-PDP (Gal-PEG-PDP). The product was confirmed by TLC (chloroform/methanol/ acetic acid 100/30/2).

C. Post-coating polyplex

The Gal-PEG-PDP was reduced by DTT similar to that of F-PEG-PDP as described in Example 3. Polyplexes were prepared by adding DNA into P25-MB or LP25-MB solution in water or 10-150 mM NaCl, at a N/P ratio (amine nitrogen of PEI vs. phosphate of DNA, molar ratio) 1-50, preferably 5-15, as described (66, 67). After addition of 1 M HEPES buffer, pH 7.5-8.0, to a final concentration of 10-20 mM, to adjust to pH 7.4-8.0, the freshly reduced ligand-PEG-SH (F-PEG-SH or Gal-PEG-SH) is added to the polyplex and reacted for 2-6 hours at room temperature or overnight at 4°C. The post-coated polyplex can be used directly or purified by Sepharose CL-4B chromatography to get rid of the unconjugated ligand-PEG. Sepharose CL-4B chromatography confirmed that more than 50% of ligand-PEG-SH was linked to the polyplex. 50% dextrose was added to a final concentration of 5% for i.v. administration.

Example 9

Ligand-PEG post-coating stabilized polyplex mediated transfection *in vitro* and *in vivo*

This example demonstrates the *in vitro* and *in vivo* transfection activity of F-PEG and Gal-PEG post-coating stabilized polyplexes.

The plasmid, pLuc, which contains the firefly luciferase gene under CMV promoter was used in the study (35). The polyplex P25-pLuc was prepared by adding P25-MB (10 mM of monomer in water, pH 7.4 by HCl) to the pLuc DNA solution while vortexing and vortexed an additional 1 minute, at a N/P ratio 6-12. The F-PEG and Gal-PEG were post-coated to the polyplex as described in Example 8. The *in vitro* transfection was performed in 24-well plates, as described in Example 4 and references 27 and 35. The transfection reagent solutions were added to the cells, JSQ-3, in the presence of 10% serum. 24 hours later, the cells were washed and lysed to measure the luciferase activity and protein concentration (35). The results are expressed as 10^3 relative light units (RLU) per μg protein in the lysate, as shown in Table 2.

Table 2

Ligand-PEG Post-coated Polyplex Mediated

Transfection of JSQ-3 Cells *In Vitro* in the Presence of Serum

	Luciferase Activity (x10^3 RLU/μg protein)	
Reagents (N/P=10)	2 μg DNA/well	4 μg DNA/well
P25-pLuc	7.1	98.4
F-PEG-P25-pLuc	34.6	134.0
Gal-PEG-P25-pLuc	54.4	341.3

The results show that the ligand-PEG post-coated polyplexes have very high transfection activity in the presence of serum, significantly higher than non-coated polyplex. Also it is worthy to note that the ligand-PEG post-coated polyplexes were much less cytotoxic than non-coated polyplex P25-pLuc, as indicated by the protein assay and observation under a microscope.

For the *in vivo* study, C57BK/6 black mice were i.v. injected with the ligand-PEG post-coated polyplexes as well as non-coated polyplexes, at 80-100 μg DNA/mouse in 0.4-0.5 ml/injection via tail vein. 24 hours later, the organs were excised and homogenized in 1X lysis buffer (Promega). The luciferase activities and protein concentrations were measured as described (35), and the results are expressed as 10^3 RLU/mg protein as shown in Table 3. The

Gal-LP25 and Gal-P25 were galactosylated PEIs prepared according to Zenta et al. (70) and Bandyopadhyay et al. (71). All polyplexes were prepared at the same N/P = 10.

Table 3

Systemic Gene Delivery by Ligand-PEG Post-coated Polyplex in C57BL/6 Mice

	Luciferase Activity ($x10^3$ RLU/mg protein)	
Solutions i.v. Injected	Liver	Lung
Gal-PEG-LP25-pLuc	661.05 ± 218.20	1.06 ± 0.06
Gal-LP25-pLuc	0.45	0.87
LP25-pLuc	0.48	11480.5
Gal-P25-pLuc	4.79	171.55

Table 3 shows that polyplexes without PEG coating deliver genes predominantly to the lungs and little to liver, spleen or other organs (data not shown). When PEIs were galactosylated, the reporter gene expression in liver was increased a little and that in lung was reduced, but not enough to be used for liver-targeted gene delivery (liver/lung = 0.5 or 0.03). However, when the polyplex was post-coated by Gal-PEG, significant reporter gene expression in the liver was observed, much more than that in the lungs (liver/lung = 624). The results demonstrated that the ligand-PEG post-coating can stabilize and shield the polyplex *in vivo* to survive the lung's first filtration effect (like other PEG-coated stealth vectors), and can deliver the carried gene selectively to the target tissue. The complexes prepared by this Gal-PEG post-coating method can deliver DNA selectively to the liver after systemic administration, and would be useful for liver-targeted gene therapy.

At DNA doses of 80-100 μg/mouse, we observed in the experiments that PEI-DNA polyplexes were very toxic to mice after i.v. injection, especially P25. All mice injected with P25 polyplex died within 12 hours. Even P25 modified by F-NHS (F-P25) or galactosylated (Gal-P25) showed similar toxicities (2/2 and 3/4 died within 12 hours, respectively). The only mouse which survived in the Gal-P25 group was severely ill and the anatomy showed an enlarged and pale liver. Histology showed liver lesions similar to that of acute hepatitis. All mice injected with F-PEG post-coated polyplexes survived and showed no sign of any of the above toxicities. The Gal-PEG post-coated polyplexes had reduced toxicity and anatomy showed no sign of liver lesions. The results demonstrated that the ligand-PEG post-coating can reduce the toxicity of polyplexes, which is promising for the clinical development of PEI-based gene delivery systems.

Example 10

Characterization of ligand-PEG for post-coating

This example shows the results of characterization of F-PEG-PDP and Gal-PEG-PDP for post-coating.

A. Ultraviolet Spectrophotogram

The purified F-PEG-PDP and Gal-PEG-PDP were scanned, before and after adding DTT, by a Beckman DU640 spectrophotometer from 200 nm to 700 nm. Figures 5A-D show the spectrophotograms of the scans. Figure 5A shows the scan of F-PEG-PDP. There is a 363 nm peak of folate and a 280 nm peak of PEG. After reduction by DTT (Figure 5B), a new peak at 342 nm arises, representing the cleaved 2-thiopyridone from the PDP group. Figure 5C shows a 240 nm peak of galactose as well as a 280 nm peak of PEG. After reduction by DTT (Figure 5D), the 342 nm 2-thiopyridone peak shows up. The results confirm that both PDP and the ligands were conjugated to PEG and the PDP was functional and can be reduced.

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WHAT IS CLAIMED IS:

1. A method of preparing a post-coated cationic vehicle for targeted delivery of a therapeutic molecule comprising the steps of:

(a) complexing said therapeutic molecule with said cationic vehicle to form a cationic vehicle-therapeutic molecule complex;

(b) conjugating a polymer with a targeting ligand to form a polymer-ligand complex wherein said polymer is conjugated to said ligand; and

(c) conjugating said preformed cationic vehicle-therapeutic molecule complex with said polymer-ligand complex wherein said polymer-ligand complex will coat said preformed cationic vehicle-therapeutic molecule complex thereby forming said post-coated cationic vehicle for targeted delivery of said therapeutic molecule.

2. The method of claim 1 wherein said cationic vehicle is a cationic liposome, a cationic polymer, polylysine, protamine or polyethyleneimine.

3. The method of claim 1 wherein said cationic vehicle comprises i) dioleoyltrimethylammonium-propane (DOTAP), dioleoylphosphatidylethanolamine (DOPE) and DOPE-maleimidophenyl butyrate (DOPE-MPB); ii) dimethyldioctadecylammonium bromide (DDAB), DOPE and DOPE-MPB; iii) DOTAP, cholesterol and DOPE-MPB; or iv) DDAB. cholesterol and DOPE-MPB.

4. The method of claim 3 wherein said cationic vehicle further comprises cholesterol.

5. The method of claim 2 wherein said cationic vehicle comprises 1-10 mole percent of a reactive linking group.

6. The method of claim 5 wherein said reactive linking group is a maleimido group.

7. The method of claim 6 wherein said maleimido group is maleimidophenyl butyrate.

8. The method of claim 1 wherein said polymer-ligand complex comprises a reactive group.

9. The method of claim 8 wherein said reactive group is a sulfhydryl.

10. The method of claim 1 wherein said ligand is a sugar, vitamin, protein, antibody or growth factor.

11. The method of claim 10 wherein said ligand is folate, galactose, peptide, polypeptide or antibody fragment.

12. The method of claim 1 wherein said ligand is folate.

13. The method of claim 1 wherein said polymer is polyethyleneglycol, polyvinylpyrrolidone, polyvinylmethylether, polyhydroxypropyl methacrylate, polyhydroxylpropylmethacrylamide, polyhydroxyethyl acrylate, polymethacrylamide, polydimethylacrylamide, polymethyloxazoline, polyethyloxazoline, polyhydroxyethyloxazoline, polyhydroxypropyloxazoline, or polyaspartamide.

14. The method of claim 1 wherein said polymer is polyethyleneglycol.

15. The method of claim 1 wherein said cationic vehicle is polyethyleneimine and wherein 0.1-25% of amines in said polyethyleneimine are bound to a sulfhydryl reacting molecule.

16. The method of claim 15 wherein DNA and polyethyleneimine are at a ratio of N to P of 1-50.

17. The method of claim 1 wherein said post-coated cationic vehicle has a diameter smaller than 80 nm.

18. The method of claim 1 wherein said post-coated cationic vehicle has a diameter in the range 35-80 nm.

19. The method of claim 1 wherein said post-coated cationic vehicle has an average diameter of approximately 60 nm.

20. The method of claim 1 wherein said ligand is covalently bound directly to said polymer.

21. The method of claim 1 wherein said therapeutic molecule is a gene, a high molecular weight DNA, plasmid DNA, an antisense oligonucleotide, a peptide nucleic acid, a chemical agent, DNA, RNA, ribozyme, CpG sequences, a viral particle, a growth factor, cytokine, an immunomodulating agent, or a protein which stimulates or suppresses the immune system.

22. A method of preparing a ligand-polymer complex capable of covalently binding with a reactive group on a cationic vehicle, comprising the steps of:
a) selecting a polymer with two ends which has a functional moiety at each of the two ends, wherein the functional moieties can be the same or different;
b) adding a chemical entity comprising a moiety capable of being converted to a reactive crosslinker a first end of said polymer of step (a);
c) adding said ligand to a second end of said polymer of step (a); and
d) converting said moiety to reactive moiety;
wherein said reactive moiety can form a covalent bond with a reactive group.

23. The method of claim 22 wherein said functional moiety is selected from the group consisting of amine, sulfhydryl, carboxyl and hydrazide.

24. The method of claim 22 wherein said chemical entity comprises a sulfur capable of being converted to a sulfhydryl and
d) converting said moiety to a moiety with a free sulfhydryl;
wherein said free sulfhydryl can form a covalent bond with a reactive group on said cationic vehicle.

25. The method of claim 22 wherein said chemical moiety is dithiopyridine.

26. A method of preparing a ligand-polymer complex capable of covalently binding with a reactive group on a cationic vehicle, comprising the steps of:

a) selecting a polymer with two ends which has a functional moiety at each of the two ends, wherein the functional moieties can be the same or different;

b) covalently bonding a ligand to a first end of said polymer;

c) adding a chemical entity comprising a reactive crosslinker to a second end of said polymer of step (b);

wherein said reactive crosslinker can form a covalent bond with a reactive group of a cationic vehicle.

27. The method of claim 26 wherein said ligand is folate.

28. The method of claim 26 wherein said chemical entity is 2-iminothiolane.

29. A cationic vehicle prepared by a method of any one of claims 1-21.

30. A polymer-ligand complex prepared by a method of any one of claims 22-28.

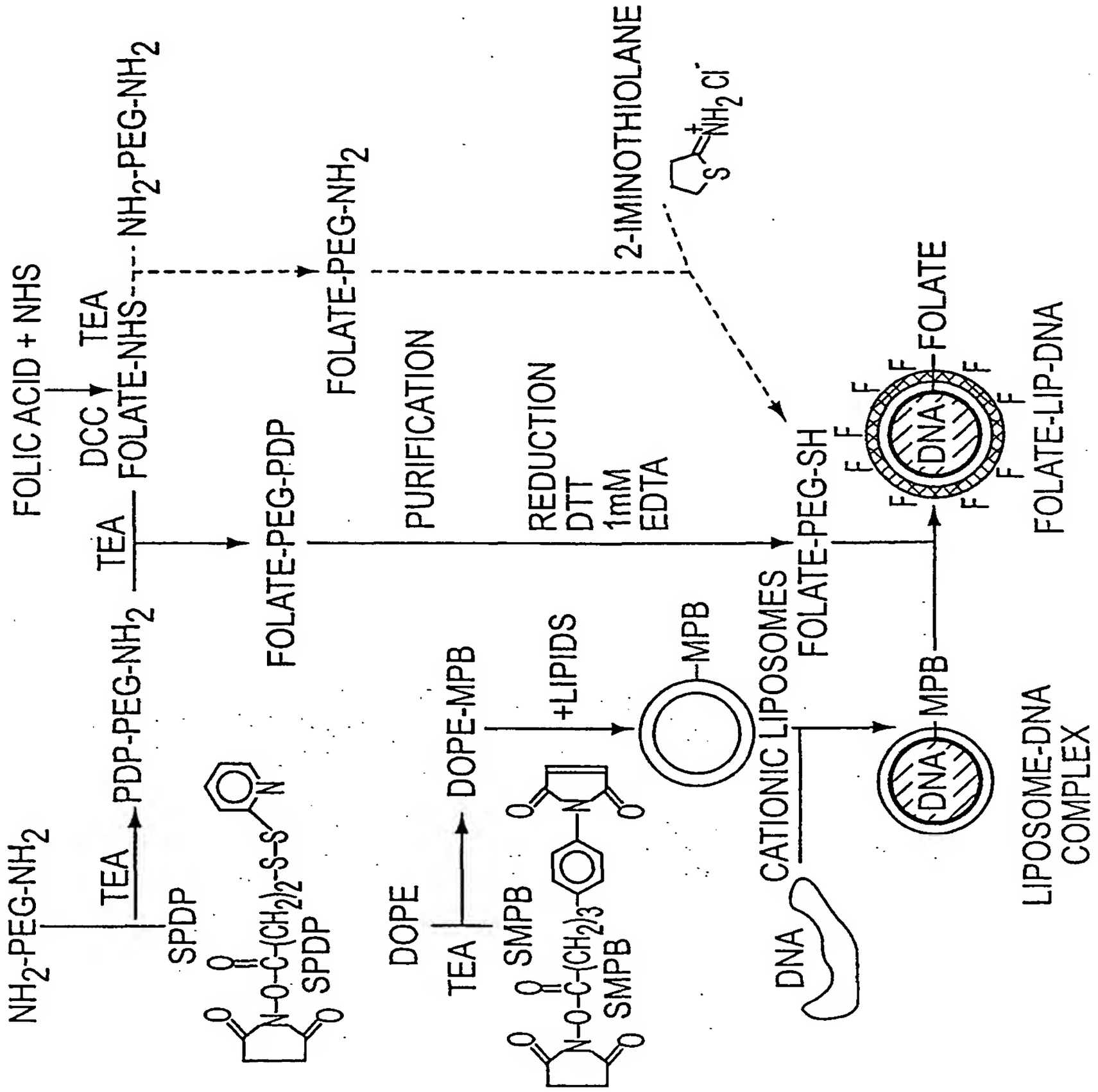


FIG. 1

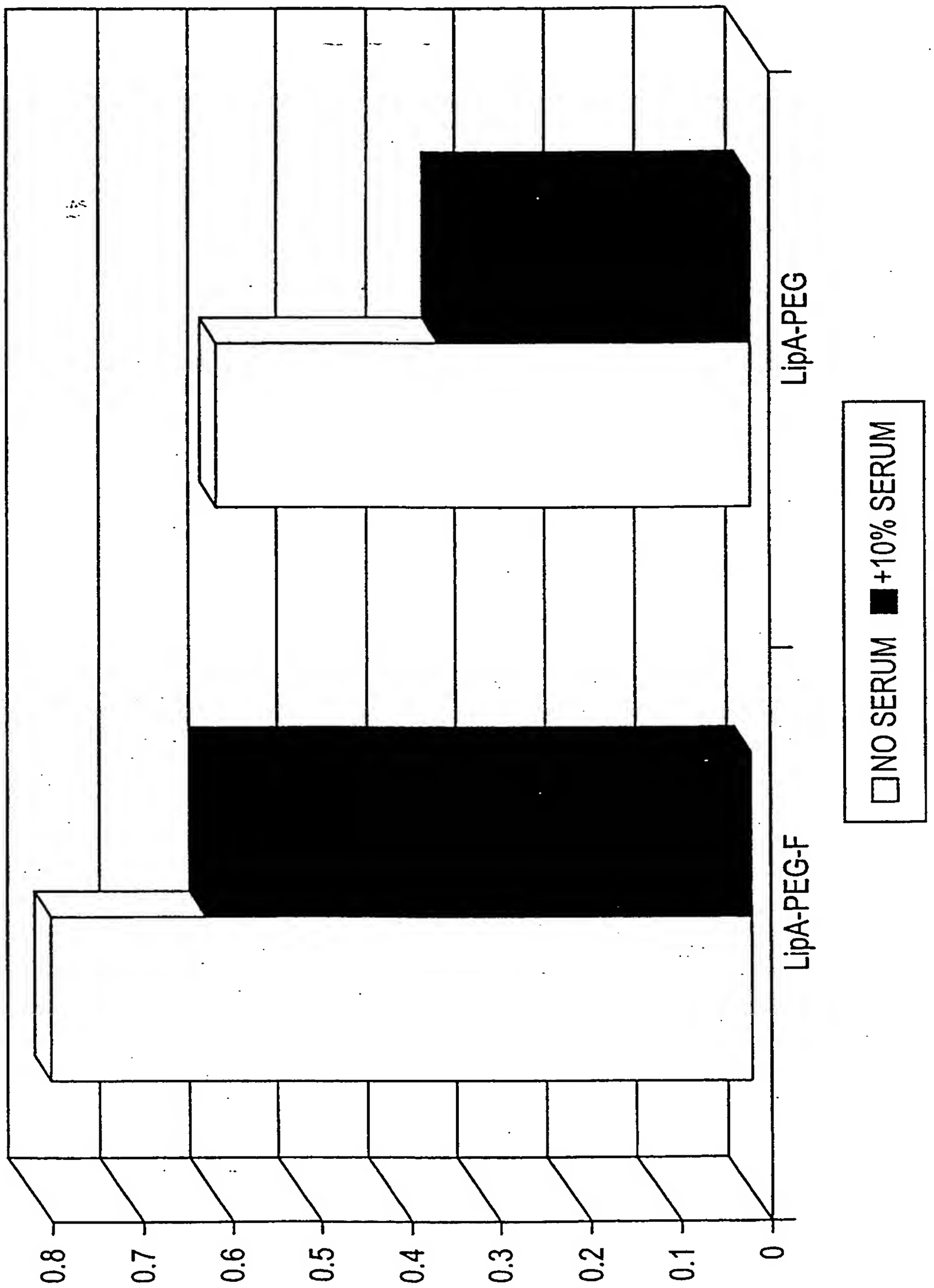


FIG. 2

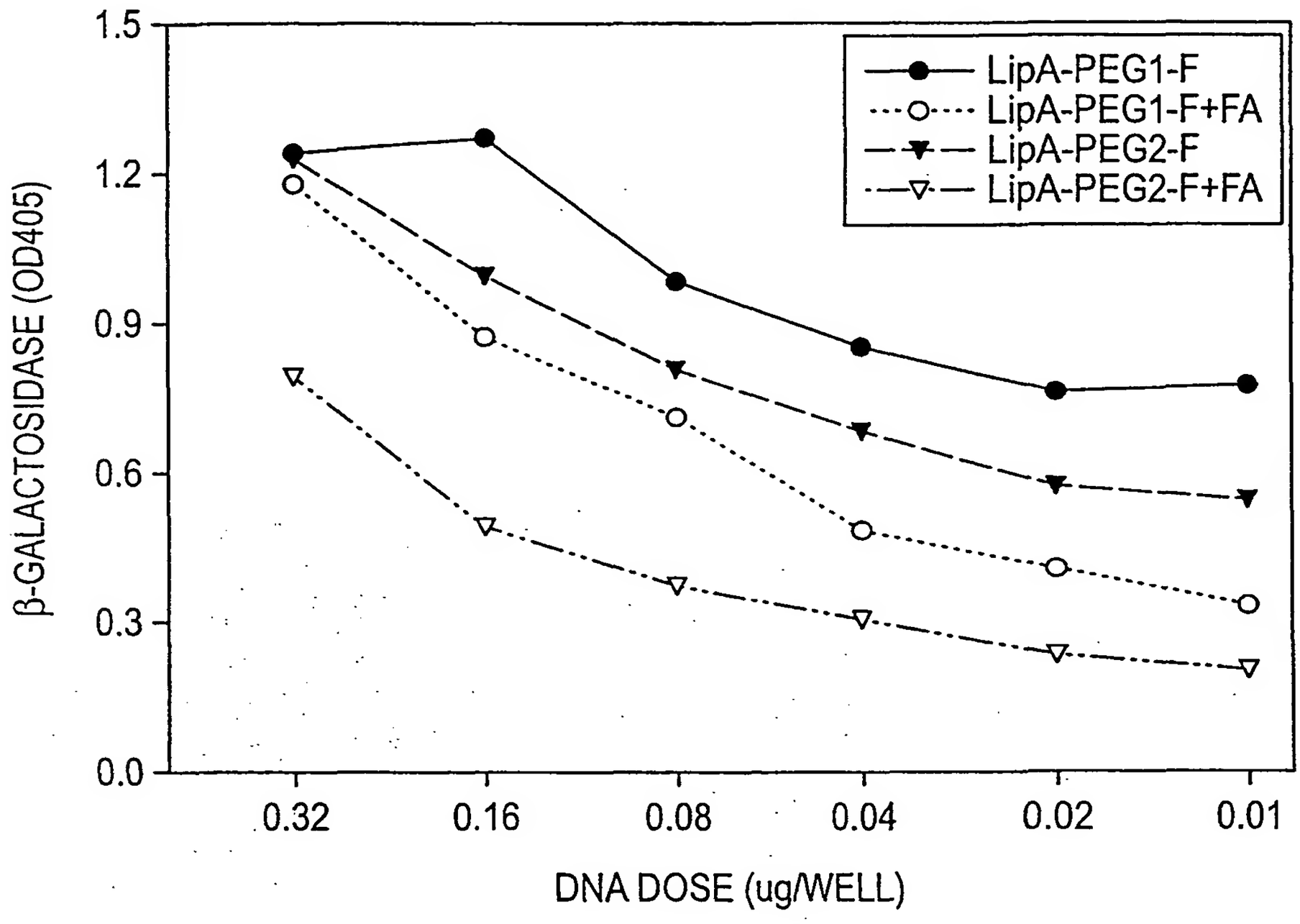
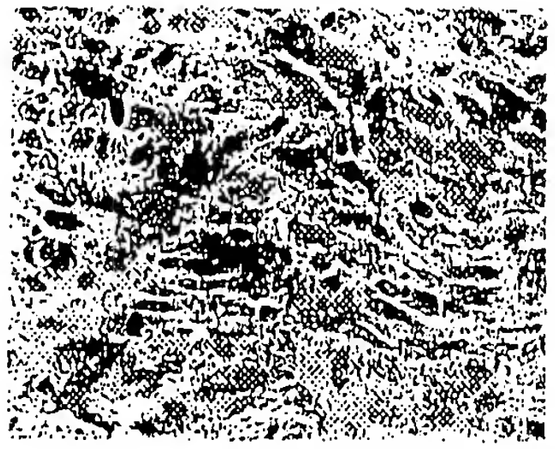


FIG. 3

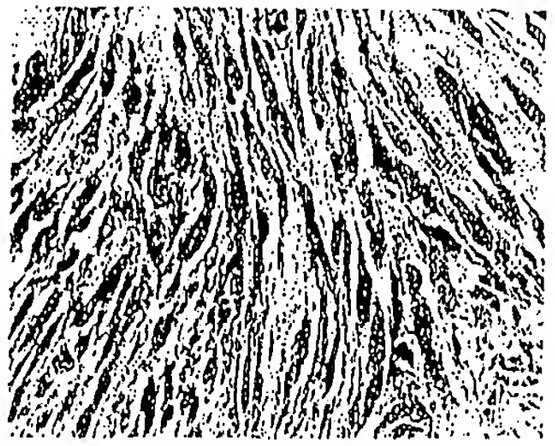
PCT/US00/01346

TUMOR



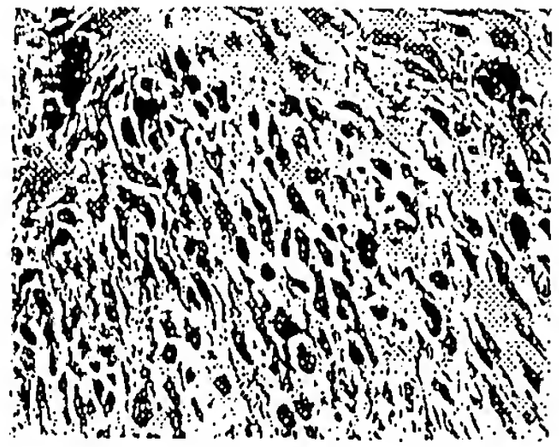
LipA-PEG1-F

FIG. 4A



LipA-PEG2-F

FIG. 4B

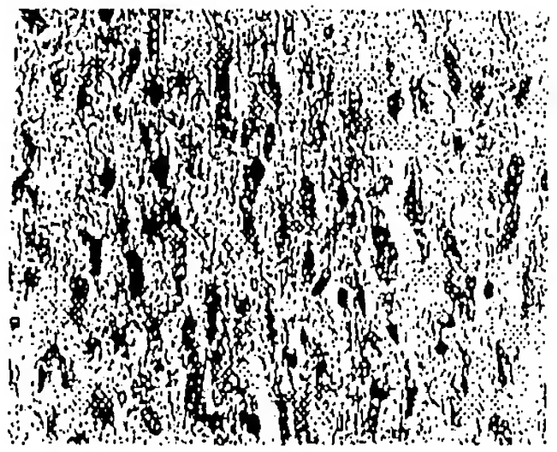


LipA-F

FIG. 4C

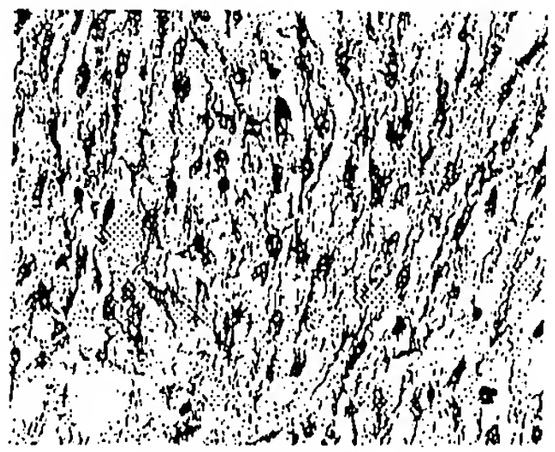
3 DAYS

TUMOR



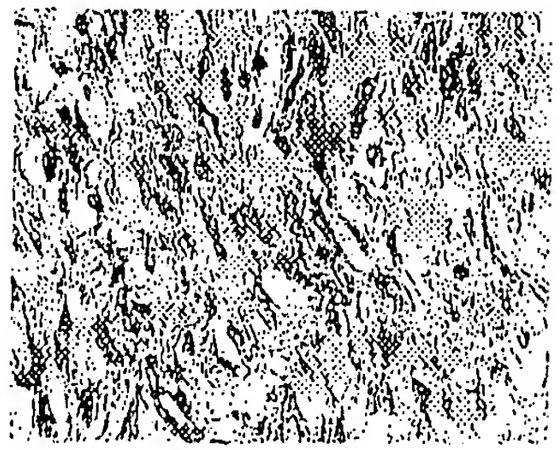
LipA-PEG1-F

FIG. 4D



LipA-PEG2-F

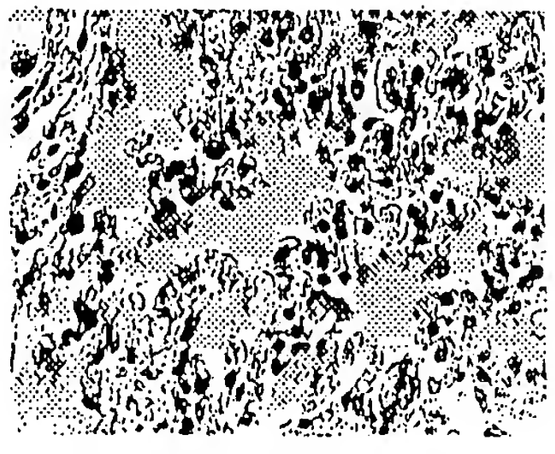
FIG. 4E



LipA-F

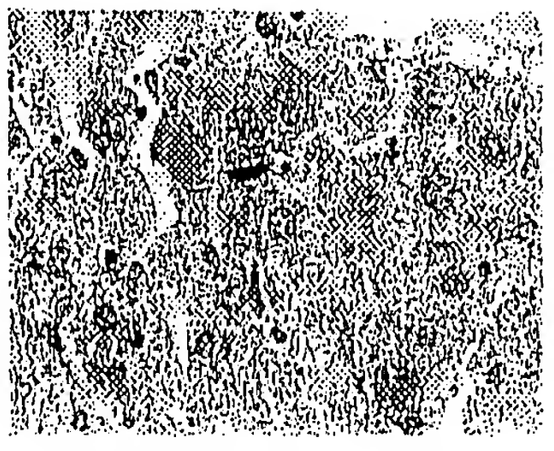
FIG. 4F

10 DAYS



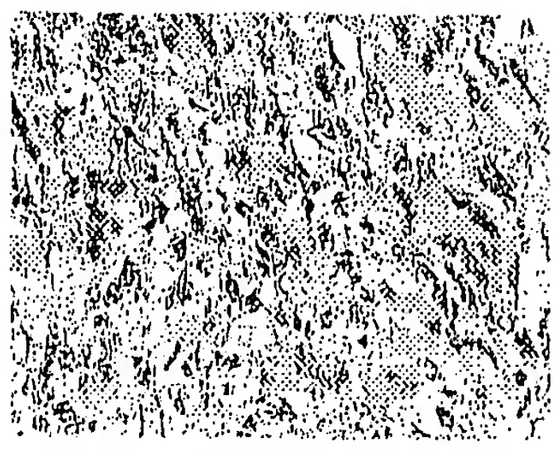
LipA-PEG1-F LUNG

FIG. 4G



LipA-PEG1-F LIVER

FIG. 4H



PLASMID ONLY TUMOR

FIG. 4I

3 DAYS

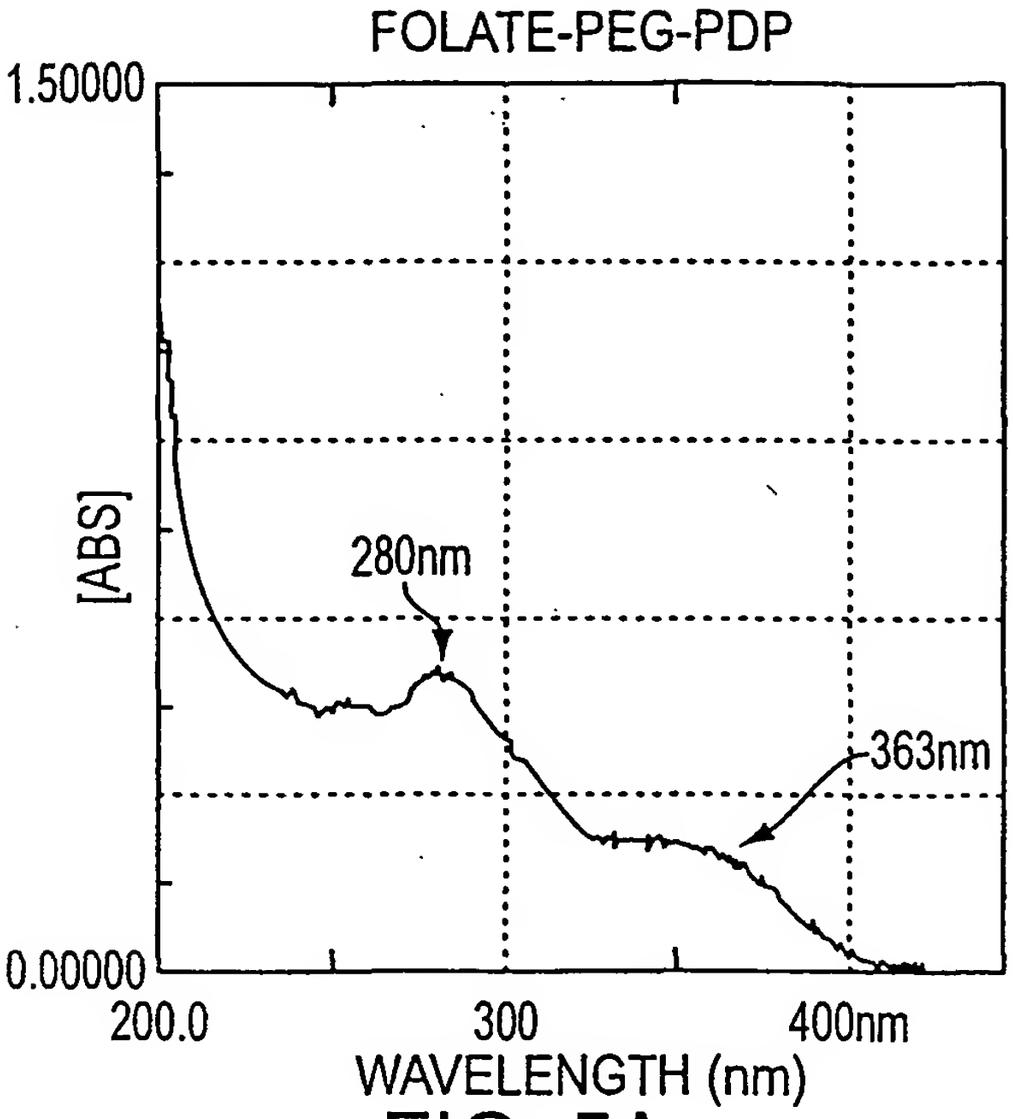


FIG. 5A

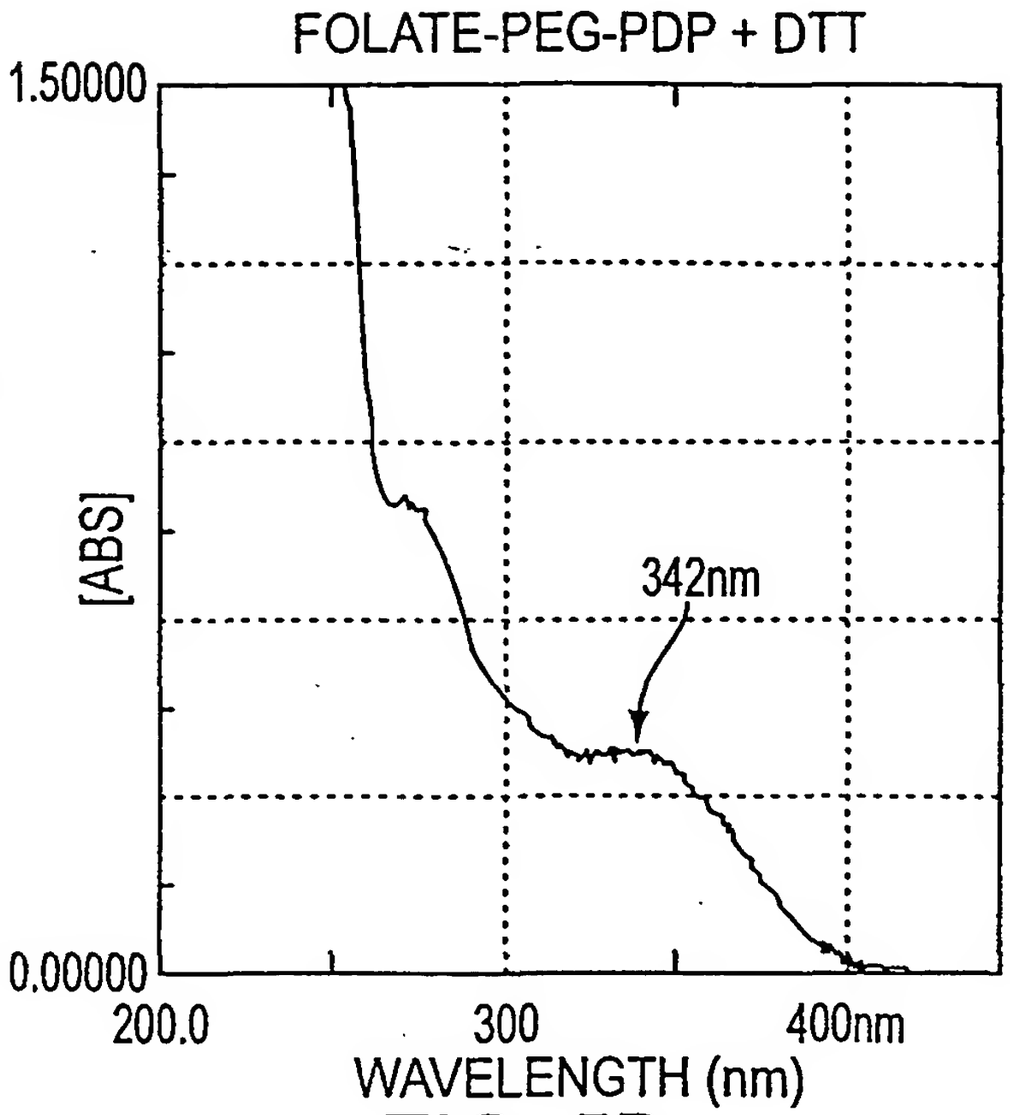


FIG. 5B

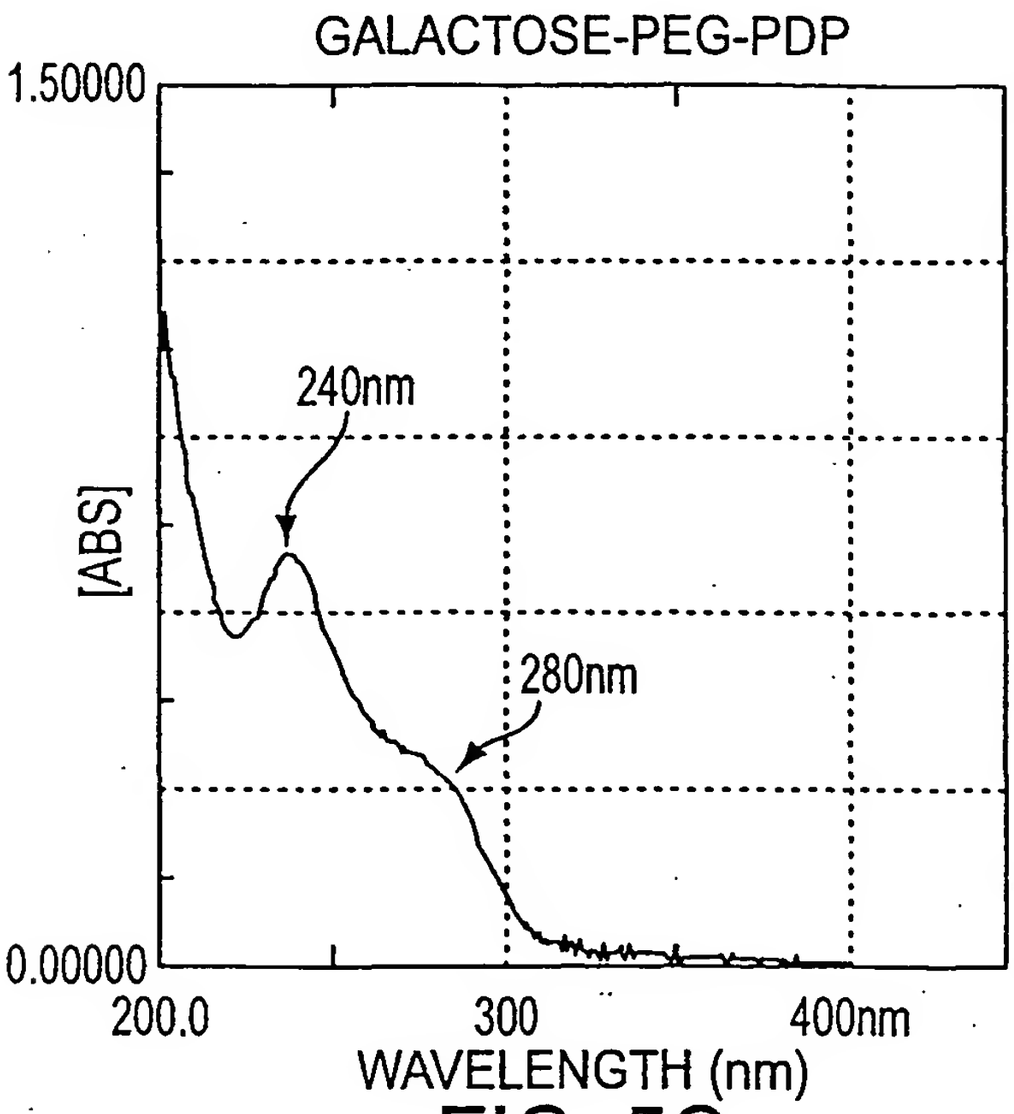


FIG. 5C

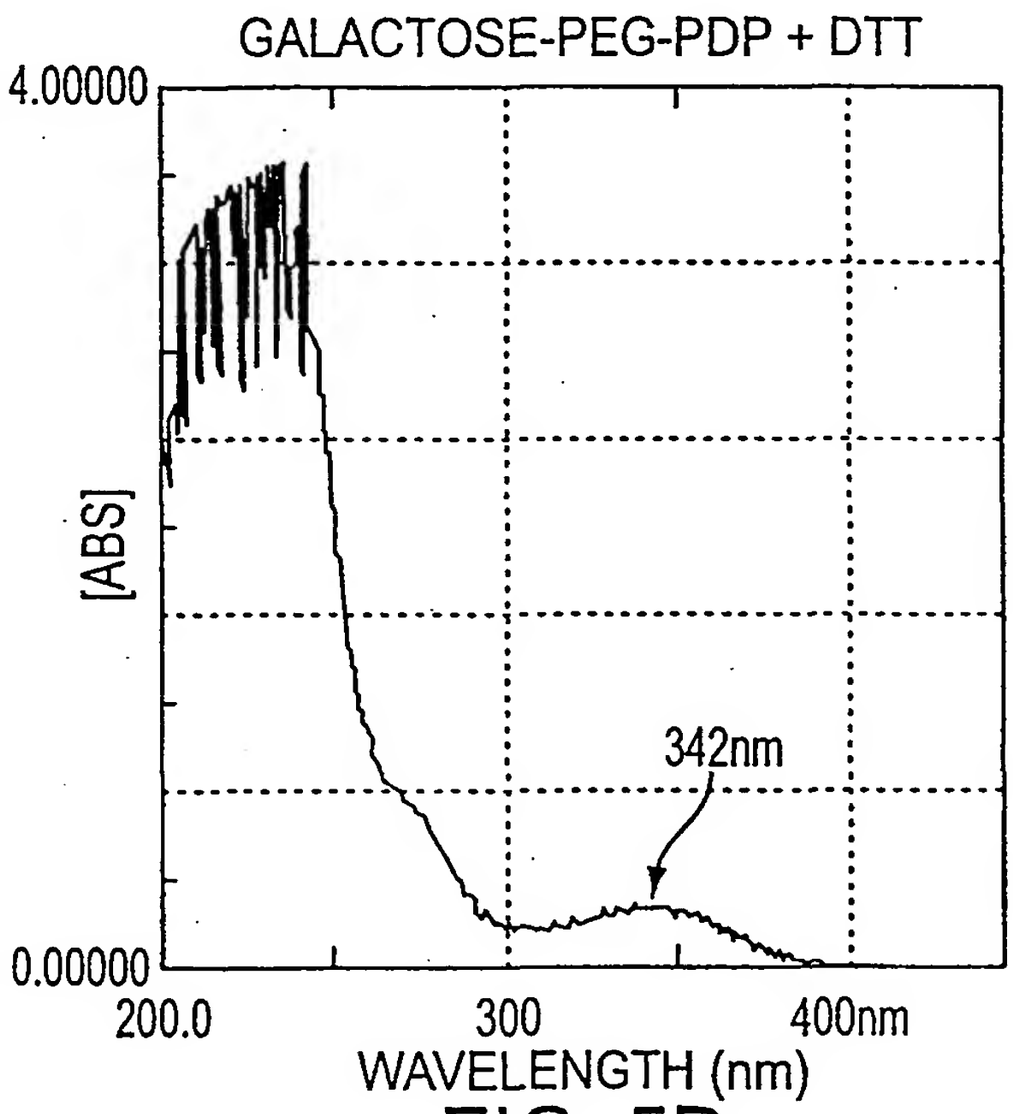


FIG. 5D

INTERNATIONAL SEARCH REPORT

Im tional Application No
PCT/US 00/01346

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HASELGRUBLER ET AL: "synthesis and applications of a new PEG derivative" BIOCONJ. CHEM., vol. 6, no. 3, 1995, pages 242-248, XP000505483 cited in the application page 243 -page 244 ---	22-28,30
X	ZALIPSKY ET AL: "PEG-grafted liposomes with oligopeptides or oligosaccharide ligands apended to the termini of the polymer chains" BIOCHEM CONJ, vol. 8, no. 2, 1997, pages 111-118, XP000886841 cited in the application page 112, right-hand column, line 15-18; figure 1 ---	26,30

-/--

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 May 2000	26/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Trifilieff-Riolo, S

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INTERNATIONAL SEARCH REPORT

Int. ational Application No
PCT/US 00/01346

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	LEE ET AL: "folate-mediated tumor cell targeting of liposome-entrapped doxorubin in vitro" BIOCHIM BIOPHYS ACTA, vol. 1233, 1995, pages 134-144, XP000607467 cited in the application page 135; figure 1	26,27,30
X	GABIZON ET AL: "targetin folate receptor with folate linked to extremities of PEG-grafted liposomes" BIOCONJ CHEM, vol. 10, no. 2, 1999, pages 289-298, XP000804255 cited in the application page 290, right-hand column, paragraph 2	26,30
X	KEEGAN-ROGERS ET AL: "targeted protection of hepatocytes from galactosamine toxicity in vivo" CANCER CHEMOTHER AND PHARMACOL, vol. 26, no. 2, 1990, pages 93-96, XP000373981 page 94, left-hand column, paragraph 1	26,30

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~CID: <WO 0043043A1 IA>

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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A61K 47/48, A61P 35/00

A1

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60/116,792 21 January 1999 (21.01.99) US

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(72) Inventors; and
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(74) Agents: SAXE, Stephen, A. et al.; Rothwell, Figg, Ernst & Kurz, Suite 701 East, 555 13th Street N.W., Columbia Square, Washington, DC 20004 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
With amended claims and statement.

Date of publication of the amended claims and statement:
21 September 2000 (21.09.00)

(54) Title: LIGAND–PEG POST–COATING STABILIZED LIPOPLEX AND POLYPLEX FOR TARGETED GENE DELIVERY

(57) Abstract

This invention relates to the area of systemic, tissue–specific non–viral gene delivery. The present invention provides a novel method to prepare ligand–directed, PEG–stabilized complex as gene delivery systems for targeted gene therapy. Due to the presence of PEG these novel complexes have longer circulating times than conventional ligand–liposome complexes. In addition, due to the presence of the ligand in the complex with PEG, these complexes are tissue targeting. Their small size further makes them very desirable for *in vivo* use.

AMENDED CLAIMS
[received by the International Bureau on 25 July 2000 (25.07.00);
original claims 22 and 26 amended; remaining claims unchanged (2 pages)]

19. The method of claim 1 wherein said post-coated cationic vehicle has an average diameter of approximately 60 nm.

20. The method of claim 1 wherein said ligand is covalently bound directly to said polymer.

21. The method of claim 1 wherein said therapeutic molecule is a gene, a high molecular weight DNA, plasmid DNA, an antisense oligonucleotide, a peptide nucleic acid, a chemical agent, DNA, RNA, ribozyme, CpG sequences, a viral particle, a growth factor, cytokine, an immunomodulating agent, or a protein which stimulates or suppresses the immune system.

22. A method of preparing a ligand-polymer complex capable of covalently binding with a reactive group on a preformed cationic vehicle-therapeutic molecule complex, resulting in the postcoating of said cationic vehicle-therapeutic complex, comprising the steps of:
a) selecting a polymer with two ends which has a functional moiety at each of the two ends, wherein the functional moieties can be the same or different;
b) adding a chemical entity comprising a moiety capable of being converted to a reactive crosslinker to a first end of said polymer of step (a);
c) adding said ligand to a second end of said polymer of step (a); and
d) converting said moiety at said first end to a reactive moiety;
wherein said reactive moiety can form a covalent bond with a reactive group.

23. The method of claim 22 wherein said functional moiety is selected from the group consisting of amine, sulfhydryl, carboxyl and hydrazide.

24. The method of claim 22 wherein said chemical entity comprises a sulfur capable of being converted to a sulfhydryl and
d) converting said moiety to a moiety with a free sulfhydryl;
wherein said free sulfhydryl can form a covalent bond with a reactive group on said cationic vehicle.

25. The method of claim 22 wherein said chemical moiety is dithiopyridine.

26. A method of preparing a ligand-polymer complex capable of covalently binding with a reactive group on a preformed cationic vehicle-therapeutic molecule complex, resulting in the postcoating of said cationic vehicle-therapeutic complex, comprising the steps of:
a) selecting a polymer with two ends which has a functional moiety at each of the two ends, wherein the functional moieties can be the same or different;
b) covalently bonding a ligand to a first end of said polymer;
c) adding a chemical entity comprising a reactive crosslinker to a second end of said polymer of step (b);
wherein said reactive crosslinker can form a covalent bond with a reactive group of a cationic vehicle.

27. The method of claim 26 wherein said ligand is folate.

28. The method of claim 26 wherein said chemical entity is 2-iminothiolane.

29. A cationic vehicle prepared by a method of any one of claims 1-21.

30. A polymer-ligand complex prepared by a method of any one of claims 22-28.

STATEMENT UNDER ARTICLE 19 (1)

The present Amendments result in Claims 22 and 26 more specifically claiming one aspect of the invention. The changes also result in the wording of Claim 22 being clearer than it was as originally submitted.